

Antifeedant and toxicity effects of thiophenes from four *Echinops* species against the Formosan subterranean termite, *Coptotermes formosanus*

Nikolas Fokialakis,^{1,2} Weste LA Osbrink,³ Leonid K Mamonov,⁴ Nadejda G Gemejeva,⁵ Amelia B Mims,³ Alexios L Skaltsounis,¹ Alan R Lax³ and Charles L Cantrell^{2*}

¹Laboratory of Pharmacognosy and Natural Products Chemistry, School of Pharmacy, University of Athens, Athens 157 71, Greece

²Natural Products Utilization Research Unit, USDA-ARS, University, Mississippi 38677, USA

³Formosan Subterranean Termite Research Unit, USDA-ARS, New Orleans, Louisiana 70124, USA

⁴Institute of Plant Physiology, Genetics and Bioengineering, Timiriazeva 45, Almaty, 05040, Republic of Kazakhstan

⁵Institute of Botany and Phytointroduction, Timiriazeva 36d, Almaty, 05040, Republic of Kazakhstan

Abstract: Over 220 crude extracts from repositories generated from plants native to Greece and Kazakhstan were evaluated for termiticidal activity against the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). Emerging from this screening effort were bioactive extracts from two Greek species (*Echinops ritro* L. and *Echinops spinosissimus* Turra subsp. *spinosissimus*) and extracts from two Kazakhstan species (*Echinops albicaulis* Kar. & Kir. and *Echinops transiliensis* Golosh.). Fractionation and isolation of constituents from the most active extracts from each of the four species has been completed, resulting in the isolation of eight thiophenes possessing varying degrees of termiticidal activity. 2,2':5',2''-Terthiophene and 5'-(3-buten-1-ynyl)-2,2'-bithiophene demonstrated 100% mortality against *C. formosanus* within 9 days at 1 and 2 wt% concentrations respectively. In addition, all but two of the eight compounds tested were significantly different from the solvent controls in the filter paper consumption bioassay.

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Keywords: *Echinops albicaulis*; *Echinops transiliensis*; *Echinops ritro*; *Echinops spinosissimus* subsp. *spinosissimus*; thiophenes; *Coptotermes formosanus*

1 INTRODUCTION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), is a major wood pest capable of tunneling through soil to locate cellulose sources, and it is currently one of the most destructive pests in the USA. Having been discovered in at least 11 states, the pest is responsible for an estimated \$1 billion annually in property damage, repairs, control and prevention.¹ The approaches used to prevent termite infestations include preventive measures such as soil-treated chemical barriers placed around wooden structures to deter termite attacks. When searching for soil termiticides, two important factors that determine the efficacy in stopping the invasion of subterranean termites are toxicity and repellency.² Either a non-repellent highly toxic substance that kills termites rapidly or a highly repellent material is required.³ Organochlorine pesticides, in particular chlordane and heptachlor, had been the soil termiticides of choice for over 40 years. These chemicals are extremely persistent

in the soil, having residual lives in excess of 25 years; however, owing to serious health and environmental concerns, manufacturers of these products voluntarily withdrew their application for reregistration with the EPA for their use in 1988.¹

This increased concern about the impact of synthetic insecticides on human and environmental health has led to interest in alternative pesticides possessing minimal risks. Furthermore, the desire for safer and more effective agrochemicals with reduced environmental and/or mammalian toxicity remains important. Essential to these efforts is the identification of new lead candidates possessing high levels of desirable biological activities, reduced unwanted toxicities, new structural types and perhaps different modes of action, thereby providing protection from cross-resistance to currently used agrochemicals.⁴

Natural product based termiticides could offer advantages in that they can sometimes be specific to a target species and often have unique modes of action with little mammalian toxicity. Nature has produced

* Correspondence to: Charles L Cantrell, USDA-ARS, Natural Products Utilization Research Unit, University, MS 38677, USA

E-mail: ccantrell@msa-oxford.ars.usda.gov

Contract/grant sponsor: ISTC K790

(Received 6 December 2005; revised version received 13 January 2006; accepted 15 March 2006)

Published online 22 June 2006; DOI: 10.1002/ps.1237

a large variety of plants with an array of survival and defensive chemical strategies. The chemical diversity found in plants is likely to yield active compounds with previously unknown modes of action. Safe pesticides with new modes of action are needed both to fight resistance development and to help meet proposed environmental regulations.⁵ Although there are a very large number of studies of the constituents of plants, relatively small percentages have been conducted through bioassay-guided isolation. Out of the thousands of compounds reported in the literature, only a small percentage were put through bioassays.⁶

In a program aimed at identifying natural termiticides as alternatives to conventional synthetic agrochemicals, more than 220 crude extracts of plants primarily collected in Greece and Kazakhstan were screened for termite activity in preliminary screens. It was clear from these preliminary studies that extracts from the roots of plants from the genus *Echinops* may yield compounds that could possess desirable termiticidal activities. The genus *Echinops* is represented by 82 species distributed in Eastern and Southern Europe, tropical and North Africa and Asia.⁷ Investigations of the genus *Echinops* have resulted in the isolation of several thiophenes⁷ which have been reported to possess many biological activities including insecticidal⁸ and fungicidal.⁹ In addition, previous phytochemical investigations of the genus *Echinops* reported on the isolation of quinoline alkaloids,^{10–11} flavanoids,¹² and sesquiterpenes¹³ as well as fatty acids and alkanes.^{14–15}

The primary objective of the present study was to identify novel, natural chemotypes from biologically active crude plant extracts that may be useful as part of termite treatment regimens in their natural form or as synthons for structure–activity studies in the future. Described below is the fractionation, isolation and biological activity of thiophenes from four separate *Echinops* species (*Echinops albicaulis* Kar. & Kir., *Echinops transiliensis* Golosh., *Echinops ritro* L., *Echinops spinosissimus* Turra subsp. *spinosissimus*). Isolated thiophenes were evaluated for activity against the Formosan subterranean termite species, *Coptotermes formosanus*.

2 MATERIALS AND METHODS

2.1 General

¹H and ¹³C NMR spectra were recorded in deuteriochloroform on a Bruker Avance 400 MHz spectrometer (Billerica, MA, USA). All ¹³C multiplicities were deduced from 90° and 135° DEPT experiments. Two-dimensional NMR techniques (COSY, NOESY, HMQC and HMBC) were performed using standard Bruker (Billerica, MA, USA) microprograms. Column chromatography was performed using a Horizon™ pump equipped with a Horizon™ flash collector and fixed-wavelength (254 nm) detector (Biotage, Inc., Charlottesville, VA, USA). Vacuum liquid chromatography (VLC) was conducted using silica gel 60H

Merck (20–40 µm). High-resolution ESI mass spectra were obtained using an Agilent 1100 HPLC coupled to a JEOL AccuTOF (JMS-T100LC) (Peabody, MA, USA). GC-MS electron ionization measurements were obtained on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS (Palo Alto, CA, USA). The GC was equipped with a DB-5 column (30 m × 0.25 mm fused silica capillary column, film thickness 0.25 µm) operated using the following conditions: injector temperature 240 °C; column temperature 60–240 °C at 3 K min^{−1}, then held at 240 °C for 5 min; carrier gas helium; injection volume 1 µL (splitless). The MS ionization energy was set to 70 eV.

2.2 Plant material

Flowering *Echinops albicaulis* was collected on 6 March 2004 in Kazakhstan in the sandhills near west Jungar Alatau. Plant aerial parts and roots were collected on bakhans (sandhills), where it was observed growing with *Eremurus inderiensis*, *Linaria pediculata*, *Limonium gmelinii*, *Artemisia terrae-albae*, *Artemisia scoparia*, *Astragalus species*, *Gypsophylla paniculata*, *Helicrysum arenarium*, *Peganum harmala* and *Corispermum* spp. A voucher specimen number 9442/18-1945 has been deposited in the Institute of Botany and Phytointroduction herbarium, Almaty, Kazakhstan.

Roots of *E. transiliensis* were collected on 12 July 2004 in Sailysky Alatau on dry slopes of mountains near Djandosov village (the Sailysky Alatau, Sugaty and Chu-Ilysky mountains are natural habitats for this species). Plants were collected in their flowering stage on dry slopes and observed growing with *Bryonia alba*, *Artemisia shrenkiana*, *A. vulgaris*, *Elytrigia repens*, *Vexibia alopecuroides*, *Salvia stepposa*, *Agrimonia asiatica* and *Marrubium vulgare*. A voucher specimen number 9442/25-1972 has been deposited in the Institute of Botany and Phytointroduction herbarium, Almaty, Kazakhstan.

Roots and aerial parts of *E. ritro* L. were collected from Attica in Central Greece on 21 July 2000 and were identified by Dr E. Kalpoutzakis. A voucher specimen is deposited in the herbarium at the Laboratory of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, University of Athens, Greece, under the number KL 017R.

Roots and aerial parts of *E. spinosissimus* subsp. *spinosissimus* were collected from the south foothills of the mountain Idi (Psiloritis) near Zaros village in Central Crete in June 1998 and identified by Dr E. Kalpoutzakis. A voucher specimen is deposited in the herbarium of the Laboratory of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, University of Athens, Greece, under the number KL 018R.

2.3 Crude extractions

Aerial parts of *E. albicaulis* (0.24 kg) were air dried followed by grinding in a Wiley Mill plant grinder. Ground plant material was extracted at

room temperature using 1.8 L of dichloromethane, providing 6.4 g of extractables after evaporation of solvent. Marc was subsequently extracted using 2.3 L of ethanol, providing 6.8 g of extractables following evaporation of solvent. Lastly, extraction with water (2.4 L) provided 33.8 g of extractables after lyophilization to remove water. Roots (0.25 kg) were extracted in an identical manner, providing 4.1 g of dichloromethane extract, 13.5 g of ethanol extract, and 25.7 g of water extract. In a similar manner, roots of *E. transiliensis* (0.53 kg) were extracted, providing 8.2 g of dichloromethane extract, 10.7 g of ethanol extract, and 41.3 g of water extract. Roots (0.37 kg) and aerial parts (0.41 kg) of *E. ritro* were extracted sequentially using dichloromethane, methanol and water (3×1.5 L for each solvent), providing 9.3, 14.7 and 48.2 g of extracts respectively for the roots and 11.9, 13.0 and 50.4 g of extracts respectively for the aerial parts. Roots (0.28 kg) and aerial parts (0.34 kg) of *E. spinosissimus* subsp. *spinosissimus* were extracted sequentially using dichloromethane, methanol and water (3×1.5 L for each solvent), providing 7.8, 16.2 and 33.0 g of extracts respectively for the roots and 13.2, 15.6 and 61.2 g of extracts respectively for the aerial parts.

2.4 Isolation of thiophenes

A portion of the *E. albicaulis* dichloromethane roots extract (1.97 g) was adsorbed to silica gel and applied to a silica gel chromatography column (40–63 μ m, 40×150 mm, 60 Å). Elution of the column was performed using increasing-polarity mixtures of hexane + ethyl acetate in a series of two linear gradient steps. Step 1 consisted of 100 + 0 to 50 + 50 using 1599 mL, with step 2 consisting of 50 + 50 to 0 + 100 using 600 mL. Finally, the column was washed with 750 mL of methanol. Column eluate was collected in 24 mL test tubes and, based on TLC similarities, recombined into 11 fractions [A, 1–14, 61 mg; B, 15–24, 21 mg (compound 4); C, 25–26, 15 mg; D, 27–36, 34 mg (compound 1); E, 37–43, 6 mg; F, 44–50, 41 mg; G, 51–55, 80 mg; H, 56–69, 41 mg; I, 70–76, 24 mg; J, 77–94, 43 mg; Wash, 698 mg].

A portion of the dichloromethane roots extract (3.03 g) of *E. transiliensis* was adsorbed to silica gel and applied to a silica gel chromatography column (40–63 μ m, 40×150 mm, 60 Å). Elution of the column was performed using increasing-polarity mixtures of hexane + ethyl acetate in a series of three linear gradient steps and finishing with a methanol wash (750 mL). Step 1 consisted of 100 + 0 to 90 + 10 using 1101 mL, with step 2 consisting of 90 + 10 to 70 + 30 using 600 mL, finishing with step 3 from 70 + 30 to 0 + 100 using 600 mL. Column eluate was collected in 24 mL test tubes (18 mm) and pooled on the basis of TLC similarities, recombined into nine fractions [A, 1–12, 74 mg; B, 13–17, 77 mg (compound 4); C, 18–22, 286 mg; D, 23–39, 130 mg (compound 1); E, 40–58, 6 mg; F, 59–66, 273 mg;

G, 67–71, 554 mg; H, 72–81, 598 mg (compound 3); I, methanol wash, 958 mg].

Echinops ritro dichloromethane extract (9.0 g) of the roots was subjected to VLC using dichloromethane + cyclohexane (10 + 90 to 100 + 0) and dichloromethane + methanol (100 + 0 to 98 + 2) gradient solutions, which finally gave ten fractions, A to J. Fraction B was subjected to silica gel column chromatography (25 \times 150 mm, 40–63 μ m, 60 Å) using a dichloromethane + cyclohexane gradient (0 + 100 to 50 + 50) and gave fractions B1 to B16. The combined fractions B2–B3 afforded compound 4 (53 mg) and fractions B5–B6 yielded compound 1 (60 mg). Fractions B8 to B12 were further fractionated by silica gel column chromatography (25 \times 150 mm, 40–63 μ m, 60 Å) using a dichloromethane + cyclohexane gradient (from 20 + 80 to 80 + 20) to afford compound 8 (18 mg). Fraction D was submitted to silica gel column chromatography (25 \times 150 mm, 40–63 μ m, 60 Å) using dichloromethane + cyclohexane gradient (from 50 + 50 to 100 + 0) and gave compound 2 (28 mg), compound 7 (12 mg) and compound 5 (23 mg). Finally, from fraction G, compound 6 (31 mg) was isolated by silica gel column chromatography (25 \times 150 mm, 40–63 μ m, 60 Å) using first dichloromethane + cyclohexane gradient (from 60 + 40 to 100 + 0) and then dichloromethane + methanol (100 + 0 to 98 + 2).

Echinops spinosissimus subsp. *spinosissimus* dichloromethane extract (7.5 g) of the roots was subjected to VLC using dichloromethane + cyclohexane (10 + 90 to 100 + 0) and dichloromethane + methanol (100 + 0 to 98 + 2) gradient solutions, which gave eight fractions, A to H. Fraction C was further fractionated by silica gel column chromatography (25 \times 150 mm, 40–63 μ m, 60 Å) using a dichloromethane + cyclohexane gradient (0 + 100 to 50 + 50) system to yield compound 4 (38 mg) and compound 1 (47 mg).

2.5 Identification of 4-[5-(penta-1,3-diynyl)thien-2-yl]-2-chlorobut-3-ynyl acetate (3; Fig. 1)

EI-MS m/z 292 (5), 290 $[M]^+$ (17), 254 (28), 230 (100), 195 (59). ^1H NMR (400 MHz): δ 7.08 (d, 1H, $J = 3.9$ Hz, H-3), 7.05 (d, 1H, $J = 3.9$ Hz, H-4), 4.92 (t, 1H, $J = 6.0$, H-3''), 4.39 (dd, 1H, $J = 6.0$ and 11.2 Hz, H-4''), 4.34 (dd, 1H, $J = 6.0$ and 11.2 Hz, H-4''), 2.09 (s, 3H, H-OAc), 2.00 (s, 3H, H-5'). ^{13}C NMR (400 MHz): δ 170.2 (s, C-OAc carbonyl), 133.7 (d, C-3), 133.1 (d, C-4), 125.0 (s, C-2), 123.1 (s, C-5), 88.4 (s, C-2''), 83.9 (s, C-4'), 66.3 (s, C-2'),* 80.1 (s, C-1''), 66.5 (t, C-4''), 80.3 (s, C-1'), 64.2 (s, C-3'),* 46.2 (d, C-3''), 20.7 (q, C-OAc methyl), 4.8 (q, C-5'),* = interchangeable carbon assignment data.

2.6 Termite bioassays

2.6.1 Experimental

Termites from four colonies of *C. formosanus* were obtained from field sites in New Orleans, Louisiana, from bucket traps,¹⁶ and maintained on spruce

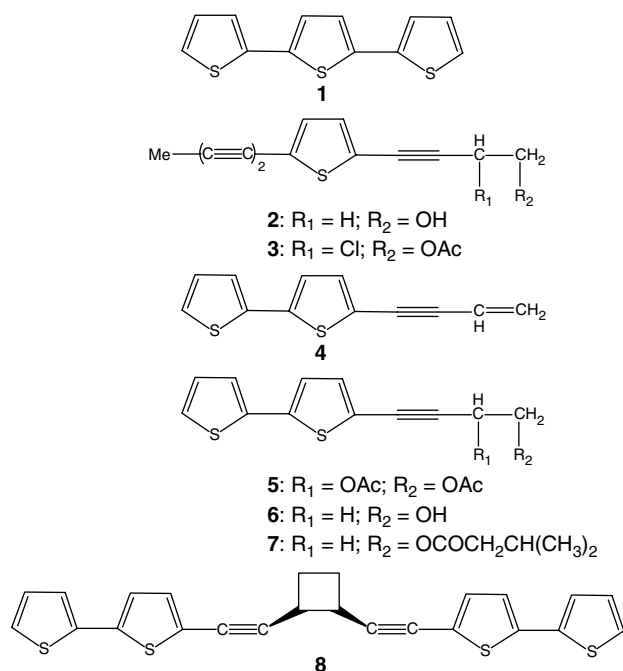


Figure 1. Structures of thiophenes isolated from *Echinops* sp.

(*Picea* spp.) slats ($10 \times 4 \times 0.5$ cm) under conditions of ca 100% relative humidity (RH) and 26 °C. Termites were identified using keys for soldier identification from Scheffrahn and Su.¹⁷

A quantity of 100 μ L of an acetone solution of the compound or fraction to be tested was blotted evenly onto a 2.5 cm diameter Whatman No. 1 filter paper. The solvent acetone was allowed to evaporate from the filter paper for several hours. The weight percentage is defined as weight of active ingredient to weight of filter paper (substrate) or weight of extract (without solvent) to weight of filter paper (substrate), whichever is appropriate. Treated filter paper disks were placed in plastic Petri dishes (35×10 mm) and moistened with 100 μ L water. Twenty *C. formosanus* workers (third instar or greater, as determined by size) and two soldiers were placed in each treatment. Treatments were replicated 4 times with termites for each replicate originating from a different *C. formosanus* colony. Petri dishes were maintained at ca 100% RH and 26 °C. Filter paper disks receiving water alone served as controls. It was previously determined that the filter paper treated with acetone solvent alone had no discernible effect on termite mortality or consumption compared with water.

2.6.2 Data analysis

Daily termite mortality was evaluated for 3 weeks. Consumption was determined by subtracting dried post-treatment from pretreatment filter paper weights. Cumulative daily mortality and consumption (mean and standard deviation) were calculated from the four replicates ($n = 20$) of each treatment. Treatments were compared using ANOVA and means separated using a protected Fisher least significant difference (LSD) test ($P < 0.05$; PROC GLM, SAS Institute,

1990). An LSD means separation test followed transformation to arcsine square root percent mortality.¹⁸ Actual percentage mortality is reported in the tables.

3 RESULTS AND DISCUSSION

3.1 Crude extract screening programs

Crude plant extract collections have been assembled in the present authors' laboratories, consisting of plants found in various regions throughout both Greece and Kazakhstan. These collections consist of at least 45 unique species of plants separated into various plant parts consisting of roots, flowers or aerial parts and extracts with solvents of various polarities and compositions (hexane, dichloromethane, ethyl acetate, methanol, ethanol, water). Over 220 different extracts from these collections were evaluated for activity against the Formosan subterranean termite species, *C. formosanus*. Emerging from this screening effort (Tables 1 and 2) were bioactive extracts from two Greek species (*Echinops ritro* and *E. spinosissimus* subsp. *spinosissimus*) and extracts from two Kazakhstan species (*Echinops albicaulis* and *E. transiliensis*).

Examination of the cumulative percentage mortality of *C. formosanus* on filter paper treated with crude extracts revealed that the highest level of activity was observed for all dichloromethane root extracts from *Echinops* species (Table 1). In fact, dichloromethane root extracts from *E. albicaulis*, *E. transiliensis*, *E. ritro* and *E. spinosissimus* subsp. *spinosissimus* all gave 100% mortality by day 14 at 2 wt% concentration. The most active of these extracts was the dichloromethane roots extract of *E. ritro*, demonstrating $97.5 \pm 5.0\%$ mortality by the third day of testing. Similarly, filter paper consumption by *C. formosanus* for *E. albicaulis*, *E. transiliensis*, *E. ritro* and *E. spinosissimus* root extracts at 2 wt% were significantly different from solvent controls. No other *Echinops* extracts from roots or any other plant part were significantly different from the controls. Purifications were performed on all four species of *Echinops* and more extensively on *E. ritro* in an effort to isolate the bioactive compounds present.

3.2 Isolation and structure determination of thiophenes from *Echinops* sp

Dichloromethane root extracts of *E. albicaulis*, *E. transiliensis*, *E. ritro* and *E. spinosissimus* were chosen for investigation in this study for reasons provided above. The dichloromethane root extract of *E. albicaulis* was subjected to silica gel column chromatography, resulting in the isolation of two major compounds, 2,2':5',2''-terthiophene (1; Fig. 1) and 5'-(3-buten-1-ynyl)-2,2'-bithiophene (4). Structure elucidation was performed by comparison of mass spectrometry and ¹H NMR data with those previously reported.^{19,20}

A portion of the *E. transiliensis* dichloromethane roots extract was purified using silica gel column chromatography techniques, resulting in the isolation of 1, 4 and 4-[5-(penta-1,3-diynyl)thien-2-yl]-2-chlorobut-3-ynyl acetate (3). Structure elucidation of

Table 1. Cumulative mortality of *Coptotermes formosanus* on filter paper treated with *Echinops* crude extracts

Experiment ^b	Species	Plant part	Extraction solvent	Mortality (%) (±SD) ^a				
				Days ^c				
				3	9	14		
1	<i>E. albicaulis</i>	Aerial	CH ₂ Cl ₂	0 A	1.3 (±2.5) C	10.0 (±12.2) BC		
			EtOH	1.7 (±2.9) A	3.3 (±5.8) C	36.7 (±50.6) BC		
			H ₂ O	0 A	3.3 (±2.9) C	30.0 (±39.1) BC		
		Roots	CH ₂ Cl ₂	5.0 (±8.7) A	98.7 (±2.9) A	100 (±0) A		
			EtOH	5.0 (±8.7) A	46.7 (±23.1) B	71.7 (±11.5) AB		
			H ₂ O	0 A	5.0 (±5.8) C	57.5 (±35.7) BC		
2	<i>E. transiliensis</i>	Untreated	n/a	0 A	0 C	6.3 (±12.5) C		
		Roots	CH ₂ Cl ₂	28.8 (±27.8) A	100 (±0) A	100 (±0) A		
			EtOH	1.3 (±2.5) B	10.0 (±4.1) B	52.5 (±12.6) B		
			H ₂ O	0 B	0 C	6.3 (±7.5) C		
		Untreated	n/a	0 B	0 C	6.3 (±12.5) C		
		3	<i>E. ritro</i>	Aerial	CH ₂ Cl ₂	1.3 (±2.5) B	1.3 (±2.5) B	1.3 (±2.5) B
MeOH	2.5 (±2.9) B				2.5 (±2.9) B	2.5 (±2.9) B		
H ₂ O	0 B				0 B	25.0 (±50.0) B		
Roots	CH ₂ Cl ₂			97.5 (±5.0) A	100 (±0) A	100 (±0) A		
	MeOH			0 B	0 B	6.3 (±7.5) B		
	H ₂ O			0 B	0 B	0 B		
Untreated	n/a			0 B	0 B	0 B		
4	<i>E. spinosissimus</i>			Aerial	CH ₂ Cl ₂	1.3 (±2.5) B	3.8 (±7.5) B	16.3 (±29.3) B
					MeOH	0 B	0 B	3.8 (±7.5) B
		H ₂ O	1.3 (±2.5) B		5.0 (±7.1) B	7.5 (±11.9) B		
		Roots	CH ₂ Cl ₂	53.8 (±21.7) A	100 (±0) A	100 (±0) A		
			MeOH	0 B	2.5 (±2.9) B	6.3 (±9.5) B		
			H ₂ O	0 B	0 B	1.3 (±2.5) B		
		Untreated	n/a	0 B	0 B	0 B		

^a For each experiment, means within a column/treatment with the same letter are not significantly different, LSD: $P < 0.05$.

^b 2 wt%; 20 workers (≥third instar)/two soldiers per rep.; four reps, four colonies.

^c Mortality measured on days 3, 9 and 14 for *E. albicaulis* and *E. transiliensis*, on days 3, 7 and 14 for *E. ritro* and on days 5, 9 and 15 for *E. spinosissimus*.

compounds **1** and **4** were performed as described previously,^{19,20} while both ¹H and ¹³C NMR spectroscopic data were lacking in the literature for compound **3**. Gas chromatography-mass spectrometry analysis of compound **3** revealed the presence of a parent ion peak m/z 290 [M]⁺ as well as a strong fragment ion at m/z 230, possibly corresponding to [M–acetate]⁺. ¹H NMR spectral analysis also supported the presence of an acetate group due to a three-proton singlet located at δ 2.09 (OAc-methyl). Additional ¹H NMR signals included another three-proton singlet at δ 2.00 (H-5'), two aromatic proton doublets at δ 7.08 (H-3) and δ 7.05 (H-4) and three aliphatic protons at δ 4.92 (H-3''), δ 4.39 (H-4'') and δ 4.34 (H-4'). Inspection of the ¹³C NMR and DEPT spectra indicated the presence of one acetate carbonyl, two aromatic doublets, two aromatic singlets, six alkyne singlets, one deshielded methine, one deshielded methylene and two methyl quartets, one of which was highly shielded at δ 4.8 corresponding to C-5'. This information suggested the presence of a thiophene ring substituted at both C-2 and C-5, where the substituents are likely to contain three alkyne groups. Careful analysis of COSY, HMQC and HMBC spectra established the

structure as that of 4-[5-(penta-1,3-diynyl)thien-2-yl]-2-chlorobut-3-ynyl acetate (**3**). Compound **3** had been reported previously;²¹ however, detailed NMR assignment data have not been provided previously and details are included above.

The *E. ritro* dichloromethane roots extract was fractionated by column chromatography, yielding a total of seven compounds, **1**, **2**, and **4** to **8** (Fig. 1). Compounds **1** and **4** were identified as described above, while compound **2** was found to be 4-[5-(penta-1,3-diynyl)thien-2-yl]but-3-ynyl alcohol,²² compound **5** was 4-(2,2'-dithien-5'-yl)-2-acetoxybut-3-ynyl acetate,²³ compound **6** was 4-(2,2'-dithien-5'-yl)but-3-ynyl alcohol,²³ compound **7** was (2,2'-dithien-5'-yl)but-3-ynyl isovalerate²³ and compound **8** was isocardopatine.²⁴ All compounds were identified as described previously in the respective references or references therein. Lastly, the *E. spinosissimus* dichloromethane roots extract was also fractionated using column chromatography, providing compounds **4** and **8**.

3.3 Antifeedant and termiticidal activity of thiophenes against *Coptotermes formosanus*

All isolated thiophenes were evaluated for activity against the Formosan subterranean termite,

Table 2. Consumption by *Coptotermes formosanus* of filter paper treated with 2 wt% of *Echinops* crude extracts

Experiment	Species	Plant part	Extraction solvent	Consumption (mg) (±SD) ^c
1 ^a	<i>E. albicaulis</i>	Aerial	CH ₂ Cl ₂	23.5 (±7.8) A
			EtOH	30.6 (±12.5) A
			H ₂ O	31.5 (±5.9) A
		Roots	CH ₂ Cl ₂	4.1 (±1.2) B
			EtOH	5.2 (±2.0) B
			H ₂ O	19.9 (±5.8) A
2 ^a	<i>E. transiliensis</i>	n/a	n/a	28.5 (±12.3) A
			CH ₂ Cl ₂	9.4 (±5.2) B
			EtOH	17.6 (±4.8) A
		roots	H ₂ O	27.1 (±11.7) A
			n/a	28.5 (±12.3) A
			CH ₂ Cl ₂	41.6 (±16.4) A
3 ^b	<i>E. ritro</i>	aerial	MeOH	44.6 (±0) A
			H ₂ O	58.7 (±21.1) A
			CH ₂ Cl ₂	10.6 (±0.7) B
		roots	MeOH	51.1 (±8.6) A
			H ₂ O	50.5 (±15.2) A
			CH ₂ Cl ₂	5.8 (±3.1) B
4 ^a	<i>E. spinosissimus</i>	n/a	n/a	41.7 (±16.7) A
			CH ₂ Cl ₂	20.6 (±6.3) A
			MeOH	22.3 (±8.1) A
		aerial	H ₂ O	37.0 (±8.1) A
			CH ₂ Cl ₂	5.8 (±3.1) B
			MeOH	33.5 (±11.6) A
		roots	H ₂ O	31.6 (±7.3) A
			CH ₂ Cl ₂	5.8 (±3.1) B
			MeOH	33.5 (±11.6) A
	Untreated	n/a	n/a	25.8 (±10.0) A

^a 20 workers (≥ 3rd instar)/2 soldiers per rep. 4 reps., 4 colonies.^b 20 workers (≥ 3rd instar)/1 soldiers per rep. 4 reps., 4 colonies.^c For each experiment, means within a column/treatment with the same letter are not significantly different, LSD: *P* < 0.05.

C. formosanus. Compounds were evaluated at concentrations between 2 and 0.5 wt%, which varied owing to quantities available for particular compounds. Analysis of the percentage mortality data by day 3 suggested that the most active of the compounds tested were **1** (1 wt%) and **4** (2 wt%) with mortalities of

78.8 ± 20.2% and 100% respectively (Table 3). The remaining compounds were not significantly different from the untreated controls at the concentrations tested at this time interval.

By day 9, compounds **1** (1 wt%) and **4** (2 wt%) both demonstrated 100% mortality against *C. formosanus*. Compound **3** (2 wt%) was the only other compound having activity that was significantly different from the untreated control, with a percentage mortality of 11.3 ± 4.8. All remaining compounds tested were inactive by day 9. On the last day of testing, day 14, compounds **1** (1 wt%) and **4** (2 wt%) remained the most active of those compounds tested, with 100% mortality against *C. formosanus*. Two of the remaining compounds tested, **3** (2 wt%) and **8** (1 wt%), demonstrated significant mortality of 22.5 ± 13.2% and 68.8 ± 36.6% respectively. The remaining compounds tested, **2** and **5** to **7**, were all deemed to be inactive since percentage mortality was not significantly different from controls (Table 3).

Consumption of filter paper was determined at the conclusion of the mortality bioassay study. Data from experiment 5 indicated that all three compounds tested were significantly different from the untreated control. Compounds **1** (1 wt%), **3** (2 wt%), and **4** (2 wt%) demonstrated consumption data of 8.6 ± 3.0%, 4.7 ± 2.4% and 4.6 ± 1.9% respectively (Table 3). Data from experiment 6 suggested that compound **2** (1 wt%) had the highest level of consumption activity, followed by that of compounds **7** (1 wt%) and **8** (1 wt%). Compounds **5** (1 wt%) and **6** (0.5 wt%) were not significantly different from the untreated control (Table 3).

It has been demonstrated previously by Osbrink *et al.*⁶ in a bioassay identical to that used in the present study that synthetic insecticides showed 100% mortality at levels of ≤0.01%. In particular, permethrin and cypermethrin gave cumulative mortalities of 100% by day 1 at 0.01 wt%. Comparing this with activity reported for isolated thiophenes **1** and **4** (Table 3), it

Table 3. Cumulative mortality of *Coptotermes formosanus* on filter paper treated with isolated thiophenes, and filter paper consumed

Experiment ^a	Compound	Concentration (wt%)	Consumption (mg) (±SD)	Mortality (%) (±SD) ^b		
				Days		
				3	9	14
5	1	1	8.6 (±3.0) B	78.8 (±20.2) B	100 (±0) A	100 (±0) A
	3	2	4.7 (±2.4) B	5.0 (±7.1) C	11.3 (±4.8) B	22.5 (±13.2) B
	4	2	4.6 (±1.9) B	100 (±0) A	100 (±0) A	100 (±0) A
	Untreated	n/a	28.5 (±12.3) A	0 C	0 C	6.3 (±12.5) C
6	2	1	4.1 (±2.2) C	1.3 (±2.5) A	18.8 (±12.5) A	27.5 (±16.6) B
	5	1	19.4 (±4.7) AB	0 A	0 A	15.0 (±23.8) B
	6	0.50	26.9 (±11.9) A	0 A	0 A	1.3 (±2.5) B
	7	1	6.6 (±1.5) BC	1.3 (±2.5) A	10.0 (±16.8) A	17.5 (±22.2) B
	8	1	11.8 (±4.1) BC	0 A	23.8 (±27.5) A	68.8 (±36.6) A
	Untreated	n/a	28.5 (±12.3) A	0 A	0 A	6.3 (±12.5) B

^a 20 workers (≥ third instar)/two soldiers per rep.; four reps, four colonies.^b For each experiment, means within a column/treatment with the same letter are not significantly different, LSD: *P* < 0.05.

is clear that 100% mortality can be obtained from the use of these natural compounds at concentrations of 1 and 2% respectively.

4 CONCLUSIONS

Clearly, this study has progressed from a screening program designed to identify potential natural termite treatment approaches to the identification and selection of bioactive crude plant extracts. Thorough investigations were performed on all four species of plant identified, which led to the isolation of eight thiophene derivatives with various activities. It is clear that compounds **1** and **4** were the most effective compounds at killing termites, while compounds **1** to **4** and **7** and **8** may all work well as feeding deterrents.

The plate-type bioassays conducted in the present study are not predictors of how a compound will perform in the role of protecting a structure in the field; however, they do provide an indication of relative levels of innate termiticidal activity of the chemicals, as well as some indication of the relative minimum levels of the chemical necessary to kill *C. formosanus*. Stability and longevity of such natural compounds are also unknown at this point. Certainly, additional studies are in order that may look into these aspects as well as determine the structure–activity relationships among these compounds.

ACKNOWLEDGEMENTS

The authors thank Soloman Green III and Amber Callahan for technical assistance and Dr E. Kalpoutzakis from the Laboratory of Pharmacognosy and Natural Products Chemistry, School of Pharmacy, University of Athens, Athens, Greece, for the collection of plant material. Financial support from ISTC Project K-790 is greatly appreciated. NF was a collaborator via a fellowship under the OECD Cooperative Research Programme: Biological Resource Management for Sustainable Agriculture Systems.

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